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Panky, a novel photoreceptor-specific ankyrin repeat protein, is a transcriptional cofactor that suppresses CRX-regulated photoreceptor genes

Rikako Sanuki [a,b], Yoshihiro Omori [a], Chieko Koike [a,c], Shigeru Sato [a], Takahisa Furukawa [a,*]

[a] *Department of Developmental Biology, Osaka Bioscience Institute, 6-2-4 Furuedai, Suita, Osaka 565-0874, Japan*
[b] *Department of Molecular Development, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Sho-goin, Sakyo-ku, Kyoto 606-8507, Japan*
[c] *PRESTO, JST, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan*

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ABSTRACT

Neuronal gene transcription is regulated by both transcriptional activators and repressors. While the roles of transactivators in retinal photoreceptor development have been well characterized, the roles of repressors have been poorly understood. We isolated *Panky/Ankrd33*, a gene encoding an ankyrin repeat-containing protein. *Panky-A* was specifically expressed in retinal photoreceptors and the pineal gland, and its expression was directly up-regulated by the CRX transcription factor. Subcellular localization of PANKY-A was observed in the nucleus and cytoplasm. Additionally, transactivation analysis suggested that PANKY-A is a transcriptional cofactor that suppresses CRX-activated photoreceptor genes. Furthermore, we found by an electrophoretic mobility shift assay that PANKY inhibited the DNA-binding activity of CRX.

1. Introduction

The neural retina is an exquisitely sensitive light detector. The vertebrate retina is formed of six types of neurons and one type of glial cell in three cell layers: the outer nuclear layer (ONL), which contains rod and cone photoreceptors; the inner nuclear layer (INL), the location of bipolar, horizontal and amacrine interneurons, and Müller glia; and the ganglion cell layer (GCL), which harbors ganglion cells. Each retinal cell is generated from common precursors that retain the capacity to trans-differentiate into each retinal cell type both in vivo and in vitro [1,2]. It is known that this neural differentiation is controlled by the functions of both transcriptional activators and repressors [3].

We previously reported that *orthodenticle homolog 2* (*Otx2*) plays a critical role in the cell fate determination of photoreceptor cells. In the *Otx2* CKO retina, differentiating photoreceptor precursor cells are converted to amacrin-like neurons [4,5]. In terminal differentiation of photoreceptor cells, CRX, another *Otx* family transcription factor, is essential for the formation of outer segments, synaptic terminals, and phototransduction pathways [1,6]. CRX functions together with several other transcriptional cofactors [2,7–12], including NRL and NR2E3/PNR. NRL is expressed in rods in the developing retina, and transactivates the *Rhodopsin* promoter in cooperation with CRX [11,13]. The deletion of *neural retina leucine zipper* (*Nrl*) in the genome converts the developing rods to the S-opsin expressing cones, indicating the importance of NRL in rod cell fate determination [14,15]. The rod photoreceptor development also requires another transcription factor, NR2E3 which interacts with CRX [11]. NRL and NR2E3 function not only to activate rod genes, but also to repress cone genes in developing rods.

To further identify genes that may regulate photoreceptor development and functions, we performed a microarray analysis of the wild-type and *Otx2* CKO retinas [5]. We identified the *Panky* (*photoreceptor ankyrin repeat protein*) gene, which was significantly down-regulated in the *Otx2* CKO retina, in this analysis. *Panky-A*, a splicing variant form of *Panky* dominantly expressed in the retina, encodes a protein containing five repeats of the ankyrin motif. The ankyrin repeat motif consists of repeated α-helices of 30–33 amino acid residues linked by loops. The ankyrin repeat is one of the most common motifs and has also been found in various transcription factors and transcriptional cofactors [16]. In this study, we carried out histological and biochemical analyses of *Panky-A*. We found that PANKY-A is localized in the nucleus and cytosol in mammalian cells, and *Panky* expression is regulated by CRX. We also found that PANKY-A functions as a repressor for transcription of CRX-regulated photoreceptor genes.

Abbreviations: Crx, cone–rod homeobox; Otx2, orthodenticle homolog 2; Nrl, neural retina leucine zipper; Nr2e3, nuclear receptor subfamily 2 group E member 3

* Corresponding author. Fax: +81 6 6872 3933.
E-mail address: furukawa@obi.or.jp (T. Furukawa).

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2. Materials and methods

2.1. Identification of mouse Panky cDNA and phylogenetic analysis

Based on the mouse spliced EST data in the UCSC Genome Browser (http://genome.ucsc.edu), a forward primer (5′-TTGGTATCAGCTACCCCACAGA-3′) and a reverse primer (5′-GACGACCTACACAGAATACAGGTT-3′) were used for PCR amplification of a *Panky* cDNA fragment containing an entire open reading frame. The PCR reaction was carried out with KOD-Plus DNA polymerase (TOYOBO, Japan). The mouse PANKY amino acid sequence was aligned with human, bovine, chick, and zebrafish putative PANKY sequences (NP_872414, XP_001788577, XP_419009, and XP_685746, respectively) by CLASTAL-W, and the guide tree was calculated using the neighbor-joining method (Kimura's two-parameter distance method) by DNA Data Bank of Japan (DDBJ; http://clustalw.ddbj.nig.ac.jp/top-j.html).

Full methods and materials are presented in Supplementary data.

3. Results and discussion

3.1. Expression patterns of Panky

We previously screened retinal genes, which are down-stream targets of the OTX2 transcription factor, using microarray (our unpublished data). In this screen, we identified a photoreceptor-enriched gene encoding an ankyrin repeat protein with an unknown function. We referred to this gene as *Panky* (*photoreceptor ankyrin repeat protein-A*) (Genbank #FJ895380). To confirm that *Panky* expression was regulated by *Otx2*, we performed quantitative RT-PCR (Q-PCR) analysis and compared *Panky* expression in the *Otx2* CKO and control retinas. Expression of *Panky* was markedly reduced in the *Otx2* CKO retina compared to the control (Supplementary Fig. S2A). To obtain a cDNA containing a full open reading frame (ORF) of the *Panky* gene, we performed RT-PCR using cDNA synthesized from adult mouse retinal RNA, and obtained a 1.5-kb cDNA predicting an ORF of 385 amino acids in length with a 5′ in-frame stop codon (Supplementary Fig. S1). We identified two splice variants of *Panky,* and designated them *Panky-A* (a longer form) and *-B* (a shorter form). PANKY-A and -B contain five and four ankyrin repeats, respectively, in the N-terminal portion (Fig. 1A and B). The PANKY amino acid sequence is conserved from human, bovine, and chick, through to zebrafish, which display 71%, 70%, 37% and 37% identity with the mouse PANKY-A protein, respectively (Supplementary Fig. S2B). The ankyrin repeat motifs of human, bovine, chick and zebrafish PANKY display 81%, 82%, 58% and 58% identity with those of mouse PAKNKY-A protein (Supplementary Fig. S2C). Human and bovine PANKY also contains five repeats of the ankyrin motif as mouse PANKY-A dose (Supplementary Fig. S2C)**.** Human and bovine PANKY also contains five repeats of the ankyrin motif as mouse PANKY-A dose (Supplementary Fig. S2C). The chromosomal localizations of mouse and human *PANKY* genes were determined by searching the mouse and human genome databases (NCBI). Mouse *Panky* is mapped to chromosome 15F2. Human *PANKY* maps to chromosome 12q13.13. These regions do not contain a candidate mutant or disease locus so far mapped. In order to examine the tissue specificity of *Panky* expression, we performed Northern blot analysis of the *Panky* transcripts in various adult tissues. We detected a strong 1.7-kb band specifically in the retina (Fig. 1D). To examine the tissue distribution of the *Panky* variants, we examined the expression of *Panky* transcripts in the pineal gland, retina, brain and liver by RT-PCR using primers which can both amplify and distinguish the two isoforms, *Panky-A* and *-B* (Fig. 1C). We detected *Panky* expression in the retina and pineal gland where *cone–rod homeobox* (*Crx*) is also known to be expressed (Fig. 1C) [6,17]. We observed that *Panky-A* expression was predominant and *Panky-B* expression was almost undetectable in the retina. A small amount of *Panky-B* was detected in the pineal gland (Fig. 1C). We then investigated the expression patterns of *Panky* in the developing retina by section in situ hybridization (Fig. 1E–J). The *Panky* signal was first detected in the presumptive photoreceptor layer at P1 and it increased at P5 (Fig. 1G and H). A strong *Panky* signal was detected in the outer nuclear layer, which is a photoreceptor cell layer, at P9 (Fig. 1J), and then the signal diminished slightly but was significantly maintained in the adult retina (Fig. 1J). Our results indicate that *Panky* is predominantly expressed in developing and mature photoreceptors and the pineal gland. This expression pattern correlates well with that of *Crx*, *Rhodopsin* and the other photoreceptor genes at P6–P9 [18]. Around P6, photoreceptors begin to undergo terminal differentiation, forming the outer segment [19]. We therefore hypothesized that *Panky* plays a significant role in late development of photoreceptors and maintenance of mature photoreceptors. These results indicate that *Panky* is expressed at least in rods, however, it is difficult to clearly examine whether or not *Panky* is expressed in cones, because the resolution of in situ hybridization is not high enough. Previously Hsiau et al. reported the expression profiles of the *Nrl* KO and *nuclear receptor subfamily 2 group E member 3* (*Nr2e3*) KO retinas [20]. It is known that expression of many cone-specific genes is up-regulated in these KO mice, and in contrast, the expression of many rod-specific genes is down-regulated. We examined *Panky* expression in the *Nrl* KO and *Nr2e3* KO retinas, and found that Panky expression was not significantly affected between the KO and control retinas (1.4-fold in *Nrl* KO, 1.4-fold in *Nr2e3* KO). These data suggest that *Panky* is expressed in both cones and rods.

In order to further characterize the *Panky-A* protein, we raised an anti-mouse PANKY antibody. We expressed *PANKY-A* by transiently introducing a *Panky-A* expression plasmid into 661W cells, a cone photoreceptor-like cell line. We detected a PANKY-A signal of 42 kDa on cell lysates using the anti-PANKY antibody by Western blot analysis (Fig. 1K). We detected a very faint PANKY-A band in the retinal extract (data not shown). However, we failed to detect a significant signal on mouse retinal sections using the anti-PANKY antibody (data not shown). These results might be due to the low sensitivity of the anti-PANKY antibody. In order to determine the subcellular localization of the PANKY-A protein in mammalian cells, we then observed 661W cells transfected with the *Panky-A* expression plasmid by immunostaining using the anti-PANKY antibody. We co-transfected the *Panky-A* expression plasmid together with a plasmid to express either EGFP or EGFP containing an endoplasmic reticulum localization signal (ER-EGFP) into 661W cells, and immunostained transfected cells with the anti-PANKY antibody. Both EGFP and PANKY-A signals were detected in the cytoplasmic region and the nucleus (Fig. 1L–Q and U), however no PANKY-A signal was observed in the endoplasmic reticulum marked with ER-EGFP (Fig. 1R–T). To exclude the possibility that the PANKY-A signal observed in the nucleus was due to the cytosolic signal overlaying the nucleus, we examined nuclear localization of PANKY-A protein by z-stacked image analysis on the 661W cells co-transfected with both *Panky-A* and EGFP expression plasmids. We confirmed that PANKY-A signals were localized in the nucleus (Fig. 1U). To investigate the localization of PANKY-A protein in cultured retinal cells, we transfected the *Panky-A* expression vector into 661W cells and detected the PANKY protein by Western blotting. We detected PANKY-A bands in both the cytosolic and nuclear fractions, however, we detected a stronger signal in the nuclear fraction (Fig. 1V). These data showed that the PANKY-A protein was localized in both the cytosol and nucleus in cultured retinal cells.

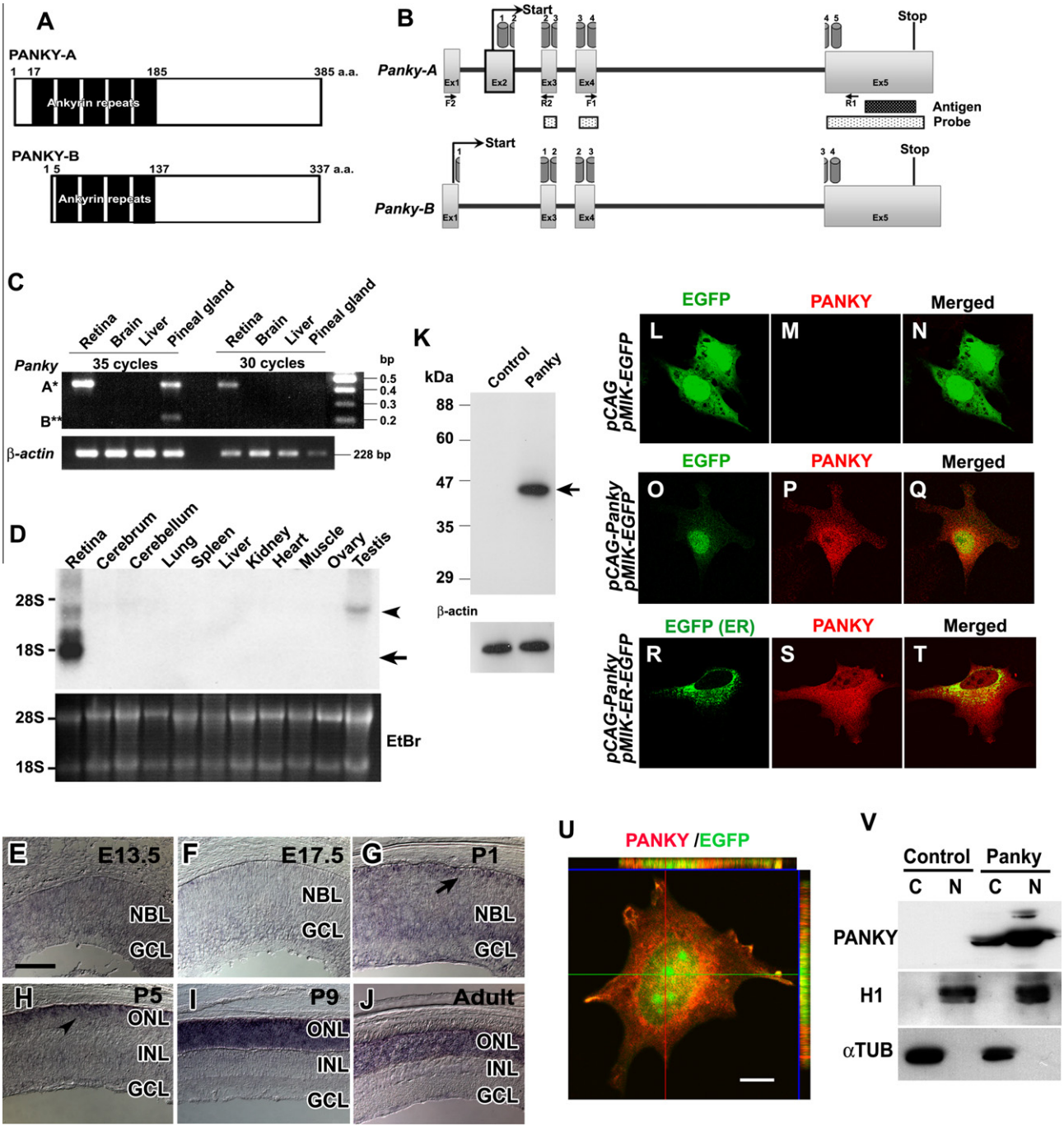

Fig. 1. Structure and expression pattern of *Panky*. (A) Schematic diagrams of PANKY-A and PANKY-B protein. PANKY-A contains five ankyrin repeats and PANKY-B has four repeats. (B) Exon–intron structures of *Panky* splice variants, *Panky-A* and *-B*. Predicted ankyrin repeats are indicated with numbers. The position of primers used for the RT-PCR were indicated (F1, F2, R1, R2). The black box shows the antigen region for the anti-PANKY antibody. The white box represents the region of the probe for Northern blots and in situ hybridization analysis. (C) Expression of *Panky* splice variants in various tissues. Primers F2 and R2 (shown in panel B) were used for the RT-PCR amplification. Single or double asterisk shows *Panky-A* or *-B*, respectively. The upper and lower lanes show PCR products amplified by primer pairs specific for *Panky* and β-actin cDNA, respectively. (D) *Panky* expression profile in adult mouse organs by Northern blot analysis. The upper panel shows the hybridization signal obtained with a mouse *Panky* cDNA probe. The arrow corresponds to 1.7-kb *Panky* transcript. The 4.5-kb band indicated by arrow head, possibly alternative spliced transcripts, has not yet been characterized. Lower panel shows EtBr staining of RNA. Five micro-grams of retinal total RNA and 10 μg of total RNA from other tissues were loaded. (E–J) *Panky* expression during development in the mouse retina. The signal was first detected in the outer aspect of NBL at P1 (arrow), and increased at P5 (arrow head). A strong *Panky* signal was detected in the ONL of the retina at P9, then the signal decreased slightly but was significantly maintained in the adult retina. NBL, neuroblastic layer; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar, 100 μm. (K) The production of PANKY antibody. Cellular proteins from the 661W cells expressing PANKY were analyzed by Western blots using the PANKY antibody. Arrow indicates the detected PANKY-A band with the molecular weight of approximately 42 kDa. Immunoblots of β-actin are loading control. (L–U) Subcellular localization of PANKY-A protein. The *pCAG* empty vector and *pMIK-EGFP*-transfected 661W cells were not stained by anti-PANKY antibody (L–N). The *Panky-A* expression vector (O–T) or empty vector (L–N) was cotransfected with an *EGFP* (L–Q and U) or *ER-EGFP* expression vector (R–T) in 661W cells, then the cells were stained with the anti-PANKY antibody. (U) Fluorescent Z-stacked optical image of 661W cell. Scale bar, 10 μm. (V) Western blot analysis of nuclear and cytosolic fractions of 661W cells. *Panky-A* expression vector (Panky) or empty vector (Control) was transfected into 661W cells. Western blot analysis of cytosolic or nuclear extract from the transfected cells was performed. PANKY-A was detected in both the cytosolic and nuclear fractions. HISTONE H1 (H1) and α-TUBULIN (αTUB) are the markers for the cytosolic and nuclear fractions, respectively. C, cytosol; N, nucleus.

3.2. Regulation of Panky-A transcription by CRX homeodomain transcription factor

Although both *Otx2* and *Crx* are expressed in developing photoreceptors, the fact that only *Crx* is highly expressed in postnatal photoreceptors and is a down-stream target of OTX2 [4] suggests that *Panky* may be transcriptionally regulated by CRX. To examine this hypothesis, we performed in situ hybridization of *Panky* mRNA on the wild-type and *Crx* KO retinas at one month (1 M) (Fig. 2A). In the *Crx* KO retina, the *Panky* transcript was dramatically reduced (Fig. 2A). We also examined *Panky* expression in the wild-type and *Crx* KO retinas by Northern blot analysis at 1 M (Fig. 2B). While expression of *Otx2* was significantly upregulated in the *Crx* KO retina, the expression of genes encoding *recoverin* and *Panky* was undetectable [6] (Fig. 2B). These data indicate that CRX is required for transactivation of the *Panky* gene. Moreover, *Panky* transcription was significantly down-regulated in the *Crx* KO pineal gland (Fig. 2C). Taken together, these data indicate that *Panky* transcription is regulated by CRX in developing photoreceptors and the pineal gland, suggesting that OTX2 regulates *Panky* expression indirectly through regulating *Crx* expression.

In order to examine whether or not CRX directly regulates *Panky* transcription, we next performed a luciferase assay using the 2.2-kb proximal promoter region of *Panky* fused to a luciferase reporter vector (*pPanky-Luc*) together with the *Crx* expression vector (Fig. 2D). This 2.2-kb region of the *Panky* upstream sequence contains six CRX/OTX2-binding consensus sequences, TAATCH (H = A, C, or T) [17,19]. As shown in Fig. 2D, the luciferase activity was significantly upregulated when the *Crx* expression vector was co-introduced with *pPanky-Luc* into HEK293 cells. To investigate the region of the *Panky* promoter responsible for transactivation by CRX, we made various deletion constructs of the *Panky* promoter region and transfected them with the *Crx* expression vector into HEK cells (Fig. 2D). We observed that the deletion of either of the two regions including putative CRX-binding sites (−2145 to −1420 bp and −967 to −124 bp) significantly decreased reporter activity. These results suggest that at least two regions (−2145 to −1420 and −967 to −124) are essential for the transactivation of the *Panky* promoter by CRX. Furthermore, to test whether the 2.1 kb fragment of *Panky* promoter is enough to drive photoreceptor-specific expression *in vivo*, we performed an *in vivo* electroporation analysis using the GFP reporter construct fused with the *Panky* promoter (Fig. 2E). We electroporated the reporter vector into the P0 mouse retina and observed the reporter expression in the P9 retina. We observed that the EGFP signals driven by the *Panky* promoter were almost exclusively localized in the ONL, whereas, the Ds-red signals driven by a ubiquitous promoter localized in all retinal cell layers (Fig. 2F–H). This result shows that the

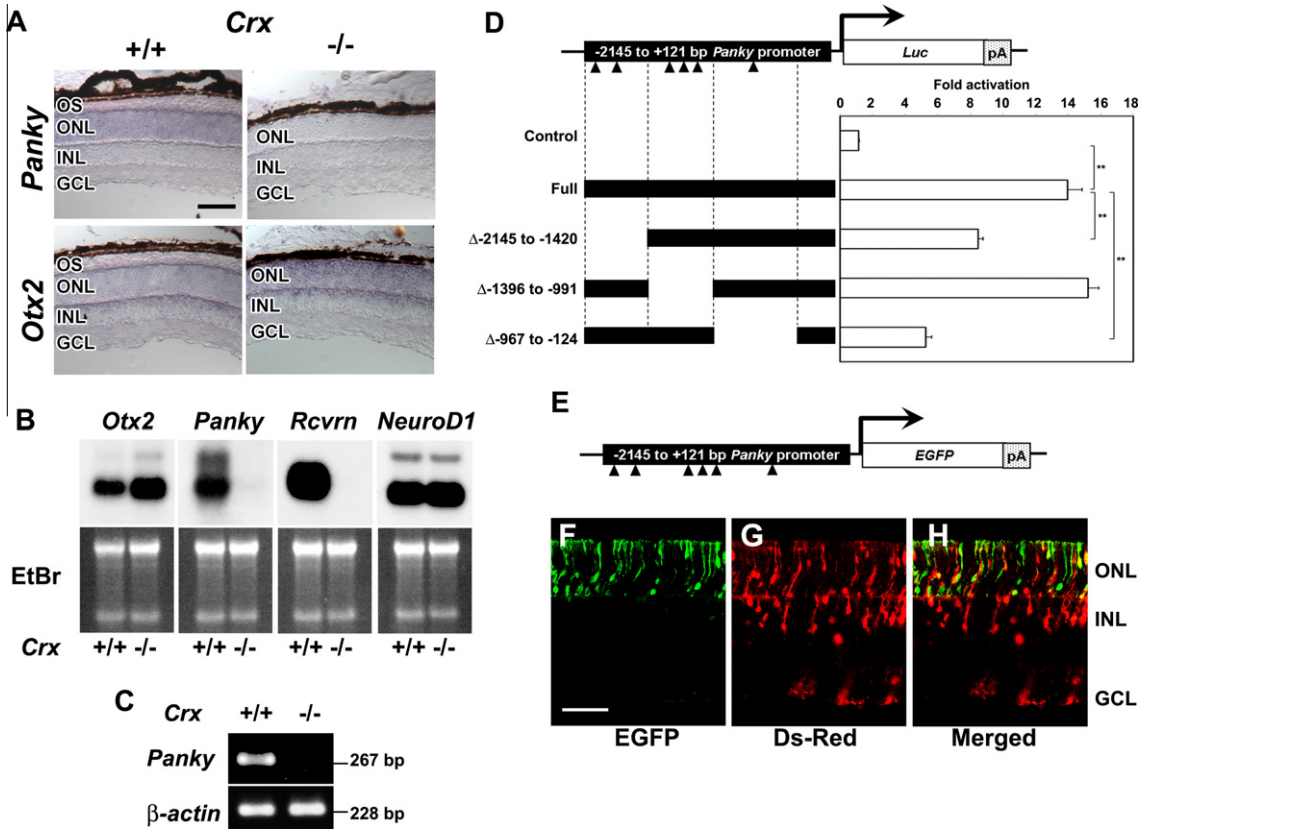

Fig. 2. *Panky* expression is regulated by CRX. (A) In situ hybridization of *Panky* and *Otx2* in the wild-type and *Crx* null mutant retina. OS, outer segment. Scale bar, 100 μm. (B) Northern blots of genes expressing in photoreceptor cells in the *Crx* null mutant retina. Upper panels are expression profiles of *Otx2*, *Panky*, *Rcvrn* (*recoverin*) and *NeuroD1*. Lower panels show their EtBr staining. (C) RT-PCR analysis of total RNAs extracted from the pineal glands in adult wild-type and *Crx* KO mice. The upper and lower lanes show PCR products amplified by primer pairs specific for *Panky* and β-actin cDNA, respectively. (D) Promoter analysis of *Panky*. The upper panel shows schematic of a *Panky* promoter. Arrowheads indicate CRX-binding consensus sequences. Various deletion constructs of the *Panky* promoter with the luciferase reporter were prepared (left panel). The *Panky* reporter plasmids or empty reporter plasmid (pGL3; Control) were co-transfected into HEK293T cells with the *Crx* expression plasmid. Internal control is a *pSV-β-galactosidase* plasmid. $^{**}P < 0.01$ by Tukey–Kramer multiple comparison test. Error bars represent standard deviation from the mean ($n = 3$). (E–H) The in vivo electroporation assay of the *Panky* promoter in the developing retina. (E) Schematic of the *Panky* promoter-EGFP reporter construct. (F–H) The P9 mouse retinal sections electroporated in vivo at P0 with both the *pPanky-EGFP* and *pCAG-Ds-Red*. The EGFP signals driven by the *Panky* promoter (F) were predominantly localized in the ONL, whereas the Ds-Red signals driven by ubiquitous promoter (G) were localized in ONL, INL and GCL. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar, 50 μm.

2.1 kb fragment of the *Panky* promoter is enough for photoreceptor-specific expression in the developing retina.

3.3. PANKY-A protein functions as a transcriptional repressor

In this study, the PANKY-A protein was demonstrated to be distributed in both the nucleus and cytosol of the 661W cells. Thus, we supposed that PANKY-A may be involved in transcriptional regulation. In order to test this possibility, we performed a luciferase assay using photoreceptor-specific gene promoters and CRX-binding consensus sequences (Fig. 3A). *Rhodopsin* transcription has been known to be transactivated by CRX and NRL in a synergistic manner [13]. We first confirmed that transfection of either *Nrl, Crx,* or *Crx* plus *Nrl* plasmids transactivated the luciferase activity of the *Rhodopsin* promoter by 4.1-, 9.2-, or 44.6-fold, respectively (Fig. 3B). We next examined the effect of *Panky-A* expression on the *rhodopsin* promoter. The luciferase activity transactivated by CRX was suppressed by PANKY-A in a dose-dependent manner (Fig. 3B). A similar suppressive effect of PANKY-A was observed for transactivation by CRX plus NRL but not by NRL alone (Fig. 3B). Transfection of 2 μg *Panky-A* plasmid exhibited 38% and 62% repression activity on transactivation by CRX and Crx plus NRL, respectively. We then examined the effect of the PANKY-A protein on the human *M-opsin* promoter–luciferase reporter plasmid. CRX transactivated luciferase activity of the *M-opsin* promoter, and PANKY-A significantly suppressed its activity by 88% (Fig. 3C). In order to test whether or not PANKY-A generally suppresses CRX transactivation activity, we then transfected a reporter vector containing a *luciferase* gene under the control of *Thymidine kinase* minimal promoter linked to five repeats of the CRX-binding consensus sequences together with the *Crx* expression vector and the *Panky-A* plasmid (Fig. 3D). CRX expression showed a significant transactivation activity on this reporter vector, and PANKY-A suppressed the CRX transactivation activity in a dose-dependent manner (Fig. 3D). These results suggest that PANKY-A functions as a transcriptional repressor for CRX-activated photoreceptor gene regulation. Although we hypothesized that PANKY-A is one of the CRX-activated gene regulators, we were not able to detect a direct interaction between PANKY-A and CRX by immunoprecipitation assay as far as we examined (data not shown).

3.4. Repression mechanism of PANKY-A

To clarify the transcriptional repression mechanism of PANKY-A on CRX-activated gene promoters, we carried out a gel-shift assay using a GST-fused homeodomain protein of CRX (GST-CRX) and

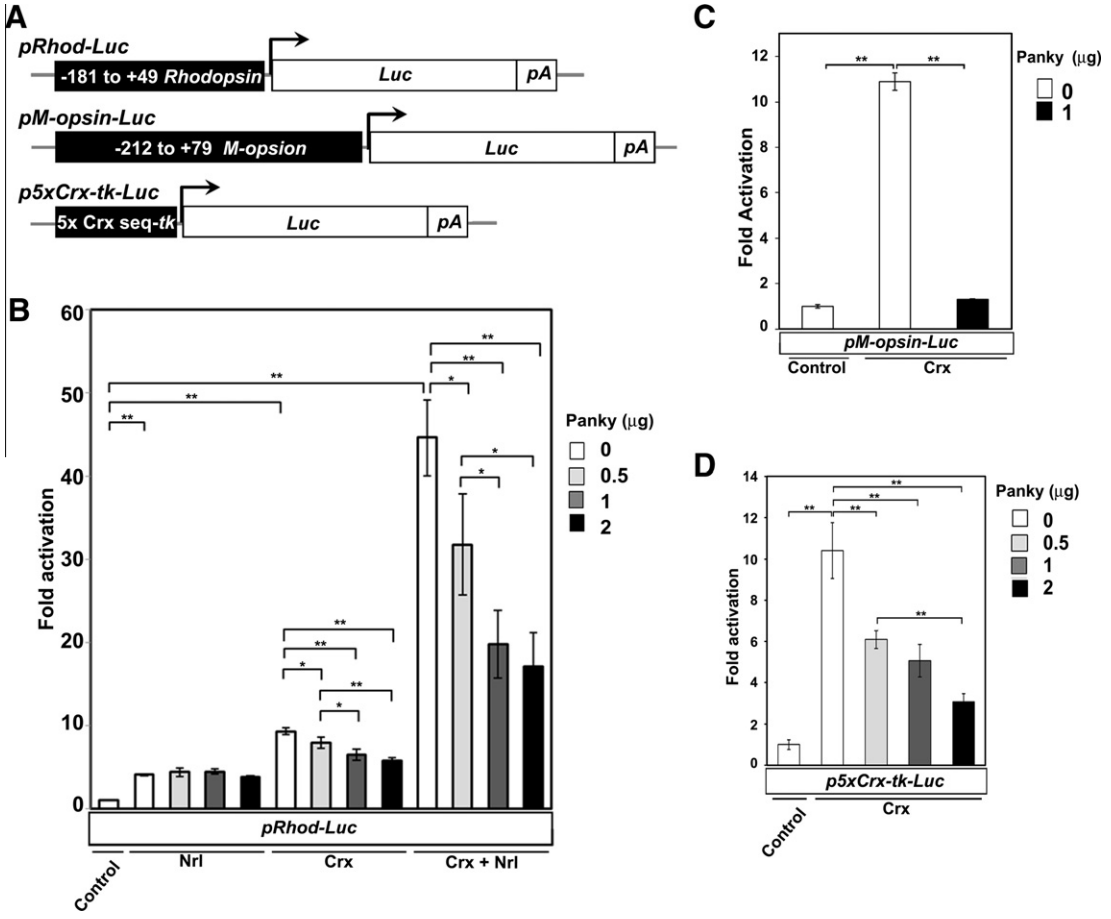

Fig. 3. Suppressive effect of PANKY-A on CRX-activated promoters of photoreceptor genes. (A) Schematic of luciferase assay promoter constructs. (B–D) Suppressive effect of PANKY-A on CRX-activated promoters of photoreceptor genes in HEK293T cells. (B) *Panky-A* plasmid was co-transfected with *Crx*, *Nrl*, or *Crx* plus *Nrl* expression plasmids, and the *Rhodopsin* promoter activation was measured by luciferase assay. (C) *M-opsin* promoter–luciferase plasmid was co-transfected with *Crx* and *Panky-A* expression plasmids, and luciferase activity was measured. (D) The *p5xCrx-tk-Luc* plasmid was co-transfected with *Crx* and *Panky-A* expression plasmids, and luciferase activity was measured. Control plasmids (empty *pMIK* or *pCAGGS*) were co-transfected with *luciferase* expression plasmids. The *pSV-β-galactosidase* expression plasmid was co-transfected, and β-galactosidase activity was used as an internal control. $^{*}P < 0.05$ and $^{**}P < 0.01$ by Tukey–Kramer multiple comparison test. Error bars represent standard deviation from the mean ($n = 3$).

in vitro translation product of *Panky-A*. We incubated ^{32}P-labeled CRX/OTX2-binding consensus sequence oligonucleotides (Otx-oligo) and GST-CRX, along with the synthesized PANKY-A protein. CRX showed a strong binding activity to the Otx-oligo, as in previous reports [1,6]. The specificity of CRX binding to the OTX consensus sequence was assessed by a competition experiment. The addition of a cold competitor to the identical oligo resulted in an inhibition of the DNA-binding activity of CRX. PANKY-A yielded an inhibition effect on the binding between GST-CRX and Otx-oligo (Supplementary Fig. S3A and B). However, we did not observe a PANKY-A and Otx-oligo complex as a shifted band. We confirmed the expression of in vitro translated PANKY-A protein by Western blots (Supplementary Fig. S3C). Although we examined if PANKY-A directly binds to the CRX consensus sequence by EMSA, we did not detect any significant binding as far as we tested (data not show). These results suggest that PANKY-A is a transcriptional cofactor suppressing CRX-activated genes through binding inhibition of CRX protein to its target DNA sequence. Retinal degeneration is a characteristic of several inherited retinal diseases such as retinitis pigmentosa (RP), macular degeneration, and Usher syndrome. It has been known that the overexpression of *rhodopsin* induces photoreceptor degeneration that is similar to many mouse models of RP. This degeneration can be induced by *Rhodopsin* levels of only 10–23% above that of the normal mouse retina [21,22]. Thus, not only transactivation but also maintenance of *Rhodopsin* expression is essential for photoreceptor cell survival. Although mechanisms of transcriptional activation in cell differentiation and maintenance have been relatively well known, mechanisms of transcriptional fine-tuning by transcriptional activators and repressors in cell differentiation and maintenance have been poorly understood. We propose that PANKY-A may play a role in the fine-tuning of CRX-activated gene expression through suppressing the DNA-binding activity of CRX in retinal photoreceptors. In a future study, targeted disruption of *Panky-A* should give us an important insight into the biological function of *Panky-A*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.12.030.

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